ANTIOXIDANT PATHWAY AS POTENTIAL THERAPY FOR ALZHEIMER'S DISEASE

by

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Introduction

Oxidative stress is the imbalance between reactive oxygen species (ROS) and antioxidants (Birben et al.,2012; Betteridge 2000; Huang., 2016). The imbalance is caused by an increase in ROS leading to the dyshomeostasis of the cellular redox balance. Both enzymatic reactions and non-enzymatic reactions can form ROS. Enzymatic reactions include phagocytosis, the respiratory chain, prostaglandin synthesis, and the cytochrome P450 system. Non-enzymatic reactions include aerobic respiration in the mitochondria. Low and moderate levels of ROS do not usually cause damage to the body but large increases in ROS can lead to cellular damage. ROS include free radicals, which are unpaired electrons that are highly reactive and unstable molecules (Birben et al., 2012; Shukla et al., 2011; Betteridge 2000).

Examples of reactive oxygen species include superoxide (O2-), hydroxyl (OH•), and hydrogen peroxide (H2O2), and are important in normal cellular processes (Scheiber et al., 2014; Halliwel., 2009). But, when they interact with metals like iron and copper, produce reactive free radicals that can attack and damage the human body (Halliwell, 2009). Endogenous production of free radicals can occur when the cell must deal with elevated O2 concentration, inflammation, infection, cancer, aging, and increased mitochondrial leakage. Pollution, radiation, heavy metals, smoking, alcohol, and dietary intake of certain compounds, add to normal cellular stress and leads to the production of even more free radicals (Poljsak et al., 2013 Pham-Huy et al., 2008; Birben et al., 2012). Excess production of these free radicals is termed oxidative stress and leads to altered cellular membranes, proteins, lipids, lipoproteins, and nucleic acid (Pham-Huy et al., 2008; Scheiber et al., 2014). To combat the increasing amounts of ROS, antioxidants

produced by our body and consumed through diet can act as the first line of defense (Pham-Huy et al., 2008; Betteridge 2000). Antioxidants help reduce ROS by either chain breaking or prevention. In the chain-breaking process, free radicals are stabilized by a chain-breaking antioxidant. In the prevention method, the antioxidant can reduce the rate of initiation of free radical production by stabilizing metal radicals like copper and iron (Pham-Huy et al., 2008). Both enzymatic and nonenzymatic antioxidants are important in blocking oxidative stress. Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), heme oxygenase-1 (HO-1), glutathione reductase, thioredoxin reductase, ferritin, and NAD(P)H: quinone oxidoreductase 1 (NQO1) (Yu et al; He et al., 2017). Non-enzymatic antioxidants include vitamin C, vitamin E, plant polyphenols, carotenoids, and glutathione (Nimse et al., 2015; Birben et al., 2012; He et al., 2017).

To mitigate oxidative damage, cells have a defense pathway known as the nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant pathway, which can detoxify, eliminate harmful chemicals, and inactivate ROS. Nrf2 is a transcription factor that plays an important role in the expression of cytoprotective genes in response to oxidative stress (Itoh et al.,1997; Taguchi et al., 2011; Schmoll et al., 2017). The Nrf2 protein consists of seven functional N2-erythroid-derived Cap ‘n’ Collar homology (Neh1-7) domains, each with different functions (He et al, 2020; Tu W et al., 2019; Yu et al., 2021). Neh 1 is a basic leucine zipper (bzip) domain that heterodimerizes with the musculoaponeurotic fibrosarcoma oncogene homolog (MAFs), another transcription factor. Neh3 is a transcriptional activation domain, while Neh 4 and Neh 5 interact with CREB binding protein which further supports transcriptional activation. Neh 6 has two motifs; DSGIS

and DSAPGS which are involved in Nrf2 degradation. Finally, the Neh 7 domain interacts with retinoic X receptor alpha (RXRα) which is a nuclear receptor that can inhibit the transcriptional activity of Nrf2 (He et al, 2020; Wang et al.,2013). The N-terminus of the Neh2 has two motifs, ETGE and DLG, which are necessary for the interaction with the Kelch-like ECH-associated protein 1 (Keap1) homodimer (Tu W et al., 2019; Taguchi et al., 2011). Keap 1 is a cysteine rich homodimer that is made up of three major functional domains; N-terminal bric-a-brac (BTB), intervening region (IVR), and C-terminal Kelch/β-propeller (Yu et al., 2021; Tu W et al., 2019; Schmoll et al., 2017; Tong et al., 2006). Under normal conditions Nrf2 is sequestered in the cytosol bound to Keap 1, and undergoes polyubiquitination, leading to its proteasomal degradation (Cullinan et al., 2004; Schmoll et al., 2017). Nrf2 binds to the Kelch domain in Keap 1 with two different interactions between the Neh2 domain and Keap 1 dimer; a high affinity binding using its ETGE motive and a low affinity binding using its DLG motif (Taguchi et al., 2011; Tu W et al., 2019; Tong et al., 2006). After Keap 1 binds to the Neh2 domain of Nrf2, its BTB domain recruits the protein Cul3, which is a component of an E3 ubiquitin ligase complex (Eggler et al., 2009; Villeneuve et al., 2010). This bridges Nrf2 to Cul3, thus allowing Keap1 to directly affect the ubiquitination of Nrf2, and its eventual degradation in the 26S proteasome (Cullinan et al., 2004). Thus, Nrf2 is constantly made and then destroyed within a cell, resulting in a half-life of fewer than 20 minutes (Zhang et al., 2006).

When a cell is under oxidative or electrophilic stress, disruption of the Keap1-Nrf2-Cul3 complex occurs by the modification of cysteine thiols of Keap1, allowing Nrf2 to translocate to the nucleus where it binds to the antioxidant response element (ARE)

and initiates the expression of Nrf2 target genes (Taguchi et al., 2011; Zhang et al., 2006). The exact mechanism by which cysteine modification leads to Nrf2 activation is not known but there are three proposed mechanisms: the Keap1-Cul3 dissociation model, the hinge and latch model, and the conformation cycling model (Taguchi et al., 2011). In the Keap1-Cul3 dissociation model, cysteine residues in Keap 1 undergo thiol modifications which leads to the disruption between Keap1 and Cul3 binding, causing Cul3 to dissociate from Keap1. This allows Nrf2 to leave the ubiquitination system (Eggler et al., 2009). In the hinge and latch model, the cysteine residues in Keap 1 undergo modification causing misalignment of lysine residues in Nrf2. Then the DLG motif of Nrf2 dissociates from Keap 1. In the conformation cycling model, the interaction between Keap1 and Nrf2 follows a cycle where there are two conformations; open when Nrf2 only interacts with a single molecule of Keap1 and closed when Nrf2 interacts with both molecules of the Keap1 homodimer. In a basal state, newly translated Nrf2 binds to one Keap 1 dimer through the ETGE motif forming the open conformation and when the DLG motif binds to the other Keap 1 dimer it forms the closed conformation. In the closed conformation, Nrf2 can be targeted for ubiquitination by the E3-ubiquitin ligase, resulting in the release of Nrf2 from Keap1 and proteasomal degradation. Now the free Keap 1 is regenerated and can bind to newly translated Nrf2 once again. When the cells are under oxidative stress, Nrf2 is not released from the closed conformation of Keap 1, and thus not targeted for ubiquitination. Newly translated Nrf2 then accumulates in the cell, leading to an influx of Nrf2 in the nucleus and increased expression of cytoprotective genes (Baird et al, 2013; Lee et al., 2020). Once in the nucleus Nrf2 heterodimerizes with the Maf protein and activates ARE dependent

gene expression (Zhang et al., 2006). ARE is a cis-regulatory element found in the enhancer region of numerous target genes encoding cytoprotective enzymes and detoxifying enzymes (Lee et al., 2004).

As previously mentioned, the antioxidant pathway is important for mitigating ROS, but increasing amounts of ROS can lead to oxidative stress, which in turn can affect many different diseases, including cancer, cardiovascular disease, and neurodegenerative disease. AD is a prevalent neurodegenerative disease that affects nearly 6 million Americans and is the 6th leading cause of death in the United States. Currently, there is no cure for this devastating type of dementia that impairs memory and mobility (Alzheimer's disease facts page, n.d.). Several risk factors are known to associate with AD, including obesity, smoking, diabetes, and hypertension. While the cause of AD is unknown, two primary hallmarks of the disease involve the formation of neurofibrillary tangles (NFTs) and Ab (Murphy et al., 2010). When tau protein is hyperphosphorylated and misfolded, it produces NFTs. Tau proteins normally stabilize microtubules and aid in the movement of axons in a healthy brain by binding to them. During cellular stress associated with AD, tau becomes hyperphosphorylated and no longer binds and stabilizes the microtubules, resulting in neurofibrillary tangles that obstruct axonal transport and result in neuronal death (Serrano-Pozo, 2011). Under normal conditions, amyloid precursor protein (APP) is cleaved by α-secretase followed by presenilin-dependent γ -secretase cleavage which produces nonamyloidogenic fragments (Shukla et al, 2011). However, certain mutations in APP or the presenilins, or extrinsic factors, such as inflammation, can cause APP to be cleaved by β-secretase, followed by presenilin-dependent γ secretase which forms Aβ (Shukla et al, 2011).

Researches have been focusing on NFTs and Ab for several decades but it is still unknown what causes their production in most patients with AD. NFTs and Ab plaques are frequently found in the cerebral cortex of a patient with AD, and as AD progresses both NFTs and Ab plaques increase (Garcia-Marin et al, 2009; Braak et al., 1991). On the other hand, researchers know that oxidative stress is a major contributor to the accumulation of Ab and hyperphosphorylation of tau. Oxidative stress is a pathological feature of AD and contributes to other biomarkers of early AD, such as the oxidation of lipids, proteins, and nucleic acids (Chen et al, 2014; Perry et al., 2002).

Phospholipids, which provide the foundation of neuronal connections, neurotransmission, and cognition, are abundant in the brain, and lipid oxidation can affect these functions (Chen et al, 2014; Huang et al., 2016). Lipid oxidation caused by ROS can disrupt the lipid bilayer and cause an increase in cellular permeability (Birben et al., 2012). Lipid oxidation also leads to the production of various lipid-derived aldehydes, including 4-hydroxy-trans-2-nonenal (HNE), which are highly reactive. In the form of glutathione conjugates, they lead to upstream activation of signaling kinases resulting in activation of the redox-sensitive transcription factors NF-kB and AP-1. This process further results in the downstream production of inflammatory cytokines, chemokines, and cellular proteins. The chronic production of these inflammatory genes plays a huge role in inflammation and apoptosis resulting in the pathogenesis of neurodegenerative diseases (Montine et al., 2002; Yadav., 2015). Oxidative stress also leads to cross-linking of proteins and changes in the electrical charge of proteins, leading to proteolysis. Lastly, oxidative stress causes DNA modification leading to DNA damage by causing either single or double-stranded DNA breaks and modifications in the pyrimidine or purines.

These DNA modifications are the main factors for cardiovascular, autoimmune and neurodegenerative diseases, as well as carcinogenesis and normal aging (Birben et al., 2012).

Despite decades of intense research, there are limited therapies and no cure for AD. Some current therapies include metal ion chelators, acetylcholine esterase (AChE) inhibitors such as donepezil, and N-methy-D-asparate (NMDA) receptor antagonists such as memantine (Cummings et al., 2014; Weller et al., 2018). Metal ion chelators have been shown to prevent protein aggregation like amyloid beta plaques. However, these compounds are known to have several side effects as it sequesters healthy metal ions in the body. (Fasae et al., 2021). AChE inhibitors enhance cholinergic neurotransmission by preventing the breakdown of acetylcholine, which is an important neurotransmitter for memory (Colović et al, 2013; Cuajungco et al., 2006). Memantine acts as a non-competitive agonist at glutamatergic NMDA receptors (Cummings et al., 2014). Glutamate, another important neurotransmitter is often elevated in AD, leading to neuronal excitability and thus blocking its NMDA receptor provides modest improvement in AD and small positive benefits on cognition and mood (Cummings et al., 2014).

The impetus for the following studies involves a collaboration with a number of labs at TCU and the University of North Texas Health Sciences Center to study potential therapeutic compounds for diseases that involve oxidative stress, such as AD. One early compound, L2 (Lincoln et al., 2013), was designed to act as an antioxidant, a metal ion chelator, and a free radical scavenger, but was subsequently found to induce an increase in cellular Nrf2 in several in vitro systems (Johnson et al., 2019). This unexpected result

made it apparent that we need to better understand the regulation of the Nrf2 pathway. Therefore, the object of the following studies was to test methods to monitor the Nrf2 pathway.

Here we used three different approaches to "follow" Nrf2 activation *in vitro* by transfecting three different plasmids into commercially available cell lines. The first plasmid contains a coding sequence for Nrf2 that is tagged by the addition of the coding sequence for a green fluorescent protein, which will help us visually locate Nrf2 by its fluorescence using microscopy. The second plasmid contains a coding sequence for luciferase that is under the control of an ARE promotor. If Nrf2 is acting as a transcription factor, it will bind to the ARE sequence in the plasmid leading to luciferase production. Adding a luciferin substrate for the luciferase enzyme will produce light that can be detected using a luminometer. The third plasmid encodes a hemagglutinin-tagged Keap1protein (Keap 1-HA) where the HA tag can be used to isolate Keap1 using commercially available antibodies to hemagglutinin and thus determine if Nrf2 is bound to Keap 1.

Materials and Methods

Cell Culture

HEK-293 cells were cultured using DMEM (Caisson Labs, Smithfield, UT), 10% FBS, penicillin-streptomycin (Sigma, St. Louis, MO), and non-essential amino acids. Cell cultures were maintained in either 10 cm dishes or T25 flask in an incubator with a temperature of 37 ºC and CO_2 at 5%. All the cells were subcloned upon reaching 60-70% confluency.

Plasmids

Bacterial stocks of each plasmid, Nrf2-GFP, ARE-Luc and Keap1-HA were purchased from Addgene (Watertown, MA). Bacterial clones were picked from LB-Agar plates, expanded, and purified using ZymoPURE plasmid kit (Zymo Research, Irvine, CA). Transfections were performed using the transfection reagent Viafect (Promega, Madison, WI). Varying ratios of plasmid and transfection reagent were tested and the ratio of plasmid to transfection reagent that produced the best level of expression was chosen. Cells were cultured in a 6-well plate at 300,000 cells/well (Nrf2-GFP and Keap1-HA) or in a 24-well plate at 25,000 cells/well (ARE-Luc).

Nrf2-GFP Expression

Transfection and TBHQ Treatment

HEK-293 cells were seeded on coverslips coated with poly-D-lysine and then transfected with 2 µg of Nrf2-GFP plasmid with 6 µL Viafect and 100 µL Opti-MEM per µg of DNA. Twenty-four hours after transfection the cells were treated with 20 µM tert-Butylhydroquinone (TBHQ, Sigma-Aldrich) overnight. Then they were either lysed the next day for western blot or visualized under the confocal microscope.

Microscopy

After the overnight treatment with TBHQ cells were fixed with 4% PFA, coverslips were sealed with AquaPolymount (Polyscience, Warrington, PA) onto slides. The GFP tag on the plasmid allows the cells to fluoresce which can be observed with a LSM 710 confocal microscope (Carl Zeiss Microscopy, White Plains, NY).

Western Blotting

After transfection, old media was aspirated, then the wells were washed with 1X PBS, which was also aspirated. Then the cells were lysed using M-PER (Thermo scientific, Rockford,IL) supplemented with phosphatase and protease inhibitors, which was added to each well and was left on ice for 5 minutes. After 5 minutes the cell lysates were collected in microfuge tubes, snap froze and placed in the -20 freezer.

A Bradford protein assay was done to measure the amount of protein present in the lysates of the cells that were transfected with Nrf2-GFP. 5 µL of each sample was loaded to a 96 well plate. The protein standard curve was made by making dilutions of 0.8 mg/mL, 0.4 mg/mL and 0.2 mg/mL from the stock concentration of 1.43 mg/mL of Gamma globulin (Bio-Rad, Hercules, CA). 5 µL of each of the standard was added to the 96 well plate. Then 250 µL of the Bradford reagent (Bio-Rad) was added to each well and was mixed thoroughly. After incubating the plate in the dark for 40 minutes, the optical density (OD) of each well was read at 595 nm using FluoStar Omega plate reader (BMG Labtech, Cary, NC).

Samples were next diluted to a concentration of 0.25 µg/µL with Laemmli buffer and lysis buffer. Proteins were denatured by boiling the samples for 5 minutes at 100 degrees Celsius. Samples were then loaded on gels. All the western equipment was purchased from Bio-Rad. After loading the samples, the gel ran at 120 V for about 1 hour. The gels were removed and put in Towbin buffer for 30 minutes while rocking. The PVDF membrane (Immobilon-P Transfer Membrane; Sigma-Aldrich, St. Louis, MO) was hydrated in MeOH for 15 seconds and placed in DI water for 2-3 minutes while rocking. Then the membrane was placed in Towbin buffer until the gels finish rocking. 4 filter

paper for each gel were submerged in ice cold Towbin buffer. Proteins were next transferred from the gel to the PVDF membrane using Trans-Blot SD (Bio-Rad) at a maximum electrical force of 18 volts and constant current of 0.3 amps per gel for 30 minutes. Membranes were then washed in Tris-Buffered Saline (TBS) and blocked in TBS-Tween 20 (TBST) containing 1% cold fish skin gelatin (Sigma-Aldrich) for 2-4 hours. After the blocking, the following primary antibodies were used for Western blotting: rabbit anti-Nrf2-R (Cell Signaling Technology, Danvers, MA) at a 1:1000 concentration and mouse anti-β-actin (Proteintech Group Inc, Rosemont, IL) at 1:75,000 concentration. Membranes were incubated with primary antibodies overnight at 4 ºC in a rocker. The next day the primary antibodies were removed and 4–5, 15 minute washes were done in TBST. Finally, appropriate secondary antibodies were added to the membrane, including goat anti-mouse-HRP at 1:10,000 and goat anti-rabbit-HRP at 1:75,000 (Jackson ImmunoResearch, West Grove, PA). Both dilutions were made in TBST. After 2 hours incubation at room temperature, antibodies were removed and membranes washed as above, followed by 2 minute incubation in Super Signal West Pico chemiluminescent substrate (Thermo Scientific, Waltham, MA). Blots were next imaged using the Syngene G-Box and GeneSys Image Acquisition Software (Syngene, Bangalore, India).

Nrf2-GFP and Keap1-HA Expression

Transfection and TBHQ Treatment

HEK-293 cells were seeded on coverslips coated with poly-D-lysine and then co-transfected with 2 µg of Nrf2-GFP plasmid and 2 µg of HA-Keap1 with 6 µL viafect and

100 µL Opti mem per µg of DNA. Twenty-four hours after co-transfection the cells were treated with 20 µM TBHQ overnight. Then they were either lysed the next day for western blot or visualized under the confocal microscope.

Immunofluorescence Microscopy

Following the overnight incubation with TBHQ cells were washed with PBST and fixed with 4% PFA. Then the cells were blocked in 2% donkey serum (Jackson ImmunoResearch, West Grove, PA) which was made in PBST for 30 minutes. After blocking, rabbit anti-HA-tag antibodies (Proteintech) at 1:500 concentration were added and left to incubate for 1 hour. After incubation the wells were washed with PBST. Finally, donkey anti-rabbit Cyc3 (Jackson ImmunoResearch) was added at 1:500 concentration and left to incubate for 1 hour, followed by PBST washes. Then coverslips were mounted on slides and sealed with AquaPolymount (Polysciences). The slides were imaged for GFP tag and HA-tag which can be observed with a LSM 710 confocal microscope (Zeiss).

Keap1-HA Expression

Transfection and TBHQ Treatment

HEK-293 cells were seeded on 6 well dishes and then transfected with 2 µg of Keap1-HA plasmid with 6 µL Viafect and 100 µL Opti-MEM per µg of DNA. Twenty-four hours after transfection the cells were treated with 50 µM and 125 µM TBHQ at both 2 hours and 6 hours. One well was treated with 125 µM TBHQ overnight. Following each treatment, old media was aspirate, then the wells were washed with 1X PBS, which was also aspirated. Then the cells were lysed and frozen as stated previously.

HA-tag immunoprecipitation

HA-tag immunoprecipitation was carried out using Pierce HA tag IP/Co-IP Kit (ThermoFisher). Following the kit protocol, for part A, 200 µL of lysate was added to spin column, followed by 20 µL of anti-HA agarose slurry which was incubated with gentle end-over-end mixing at 4 ºC overnight. The next day each spin column was washed with TBS plus 0.05% Tween 20 (TBT-T) followed by 10 seconds of centrifugation, which was repeated twice. This is followed by elution protocol 2 provided in the kit. The spin column is placed in a new collection tube. Then 25 µl of 2X non-reducing sample buffer was added to the spin column. The spin column with the collection tube was heated at 95-100 C for 5 minutes, followed by 10 seconds of centrifuge. Finally, 2 µL of β-ME was added to the 25 µL sample to prepare the sample for reducing SDS-PAGE. The sample is then analyzed by western blotting for ubiquitinated Nrf2 protein using methodology previously stated.

ARE-luciferase Expression

Transfection and TBHQ Treatment

HEK-293 cells were seeded on to a 24-well plate at 25,000 cells/well. They were subsequently transfected with 0.1 µg ARE-luc, 0.05 µg CMV-luc and 25 µL of the transfection reagent LyoVec (InvivoGen, San Diego, CA) per well. Some wells were transfected with ARE-luc, CMV-luc and different amounts of Nrf2-Myc, as indicated in the figure legends. 24 hours after transfection, cells were treated with different concentrations of TBHQ overnight and were lysed the next day for the luciferase assay.

Luciferase Assay

Luciferase assays were carried out using the Dual Luciferase Kit from Promega following the manufacturer's protocol. Briefly, cells were lysed using 1X Passive lysis buffer (Promega). The media was first aspirated, then washed with 1X PBS. Then Passive lysis buffer was added to each well and rocked for 15 minutes. After 15 minutes of rocking, the cell lysates were collected in microfuge tubes and stored at –20°C.

The lysates were thawed. A Stop and Glo (S&G) mix is made by diluting 50x Renilla luciferase substrate (Promega) in the Stop and Glo buffer (Promega). Then 20 µL of each lysate is added to new tubes. 50 µL of LARII (Promega) is added to the new tubes and Firefly luciferase activity is measured. Next 50 µL of the S&G is added to the same sample and Renilla luciferase activity is measured. These steps are repeated for all the cell lysates. The reading obtained from measuring the Firefly luciferase activity was divided by the Renilla luciferase activity to normalize for transfection efficiency. The results are presented as fold increase over control.

Results

Confocal microscopy of Nrf2-GFP transfected cells showed nuclear localization of Nrf2 in both untreated and TBHQ-treated cells (Figure 1) as visualized by the green fluorescent tag. After co-transfecting with Nrf2-GFP and HA-Keap1, confocal microscopy revealed mostly cytosolic Nrf2 localization in untreated cells, and mostly nuclear localization of Nrf2 in cells treated with 20 µM TBHQ (Figure 2). After co-transfecting with Nrf2-GFP and HA-Keap1, followed by immunofluorescence of HA tag, confocal microscopy showed orange-yellow fluorescence in the cytoplasm, suggesting some co-localization of Nrf2 and Keap1 (figure 3A). However, some yellow spots

seemed to localize to the nucleus as well. Treating these co-transfected cells with TBHQ resulted in localization of the green fluorescent Nrf2 in the nucleus while red-fluorescing Keap 1 remained in the cytoplasm (figure 3B).

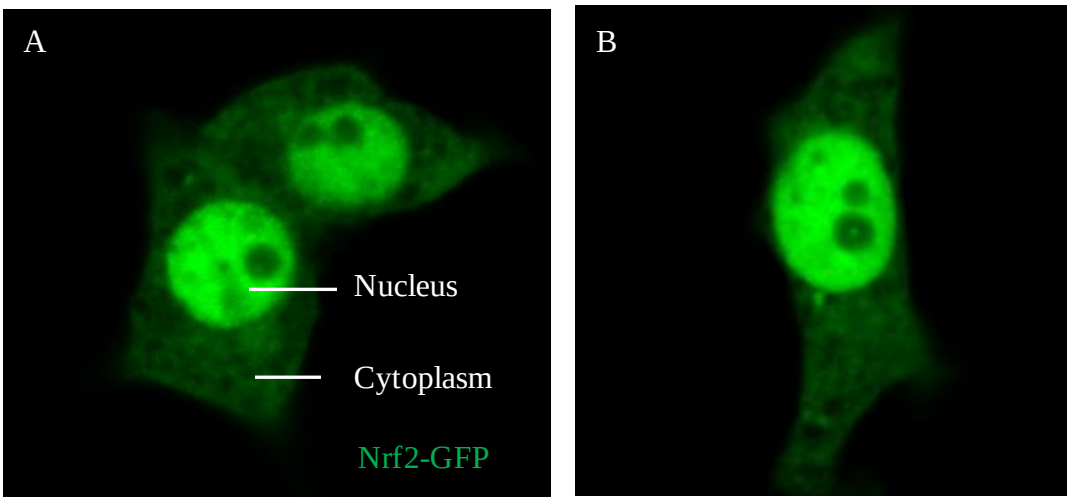


Figure 1. Overexpressed Nrf2 localizes to the nucleus in HEK cells. Cells are transfected with Nr2-GFP. Cells are visualized using the GFP fluorescence. Panel A shows untreated cells with more GFP localized in the cytoplasm. Panel B shows cells treated with 20 µM TBHQ with more GFP localized in the nucleus. Nrf2 has localized in the nucleus for both untreated and treated cells.

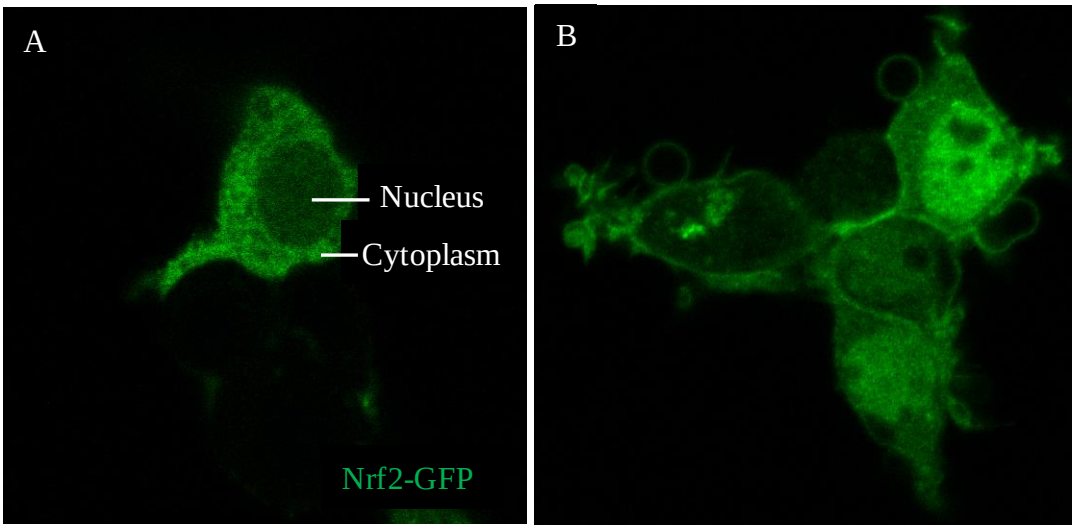


Figure 2. Co-transfection of Keap 1 with Nrf2. HEK cells were co-transfected with equal amounts of plasmid. Cells are visualized using the GFP fluorescence. Panel A shows untreated cells and panel B shows cells treated with µM TBHQ.

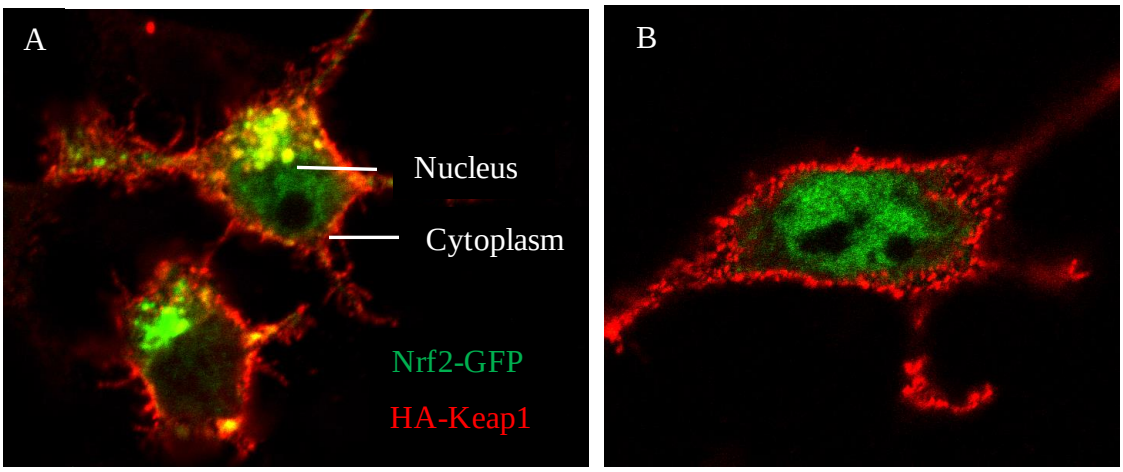


Figure 3. Co localization of Nrf2 and Keap 1. Co-transfection of Keap 1 with Nrf2 sequesters some Nrf2 in the cytoplasm as seen in panel A. While co-transfection of Keap 1 with Nrf2 sequestered Keap 1 in the cytoplasm and localized Nrf2 in the nucleus as seen in panel B. The cells were visualized using the GFP fluorescence and immunodetection of HA tag. Panel A shows cells that were untreated and panel B shows cells that were treated with 20 µM TBHQ.

Immunoprecipitation of HA-tagged Keap1, followed by a western blotting for Nrf2 has been inconclusive. In the representative Figure 4 Western blot, a faint band between 75KD and 100 KD is apparent and is believed to be Nrf2. This seems likely since Nrf2 bound to Keap1 would be ubiquitinated and thus be larger than its native 67KD size. While difficult to see, the untreated cells have a more pronounced Nrf2 band, which diminishes after 2 hours of 50 µM TBHQ treatment (Column 2). This Nrf2 band

becomes more pronounced after 6 hours of treatment. A similar pattern exists when cells were treated with 125 µM TBHQ. This showed binding interruption between Keap1 and Nrf2 in the presence of TBHQ (figure 4). It can be seen on the Western blot that there is decrease in the presence of Nrf2 in the presence of 125 µM TBHQ.

200 KD

150 KD

100 KD

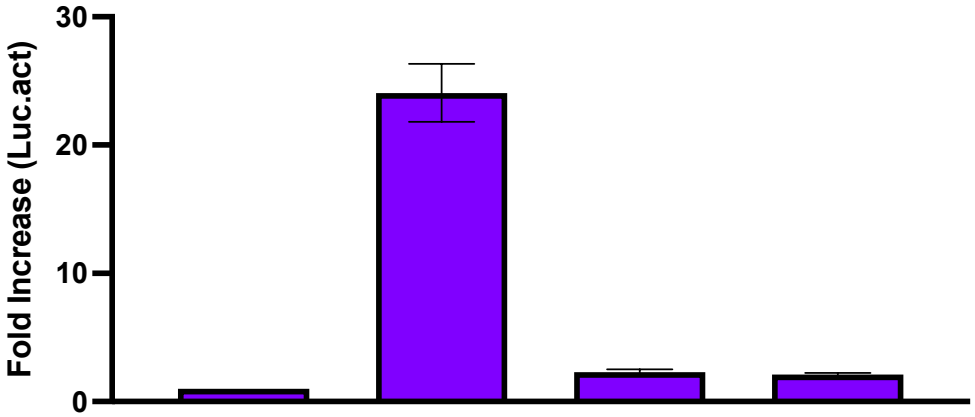
Nrf2 –

75 KD

TBHQ (µM)	–	50	50	125	125	125
Time (hr)	–	2	6	2	6	18

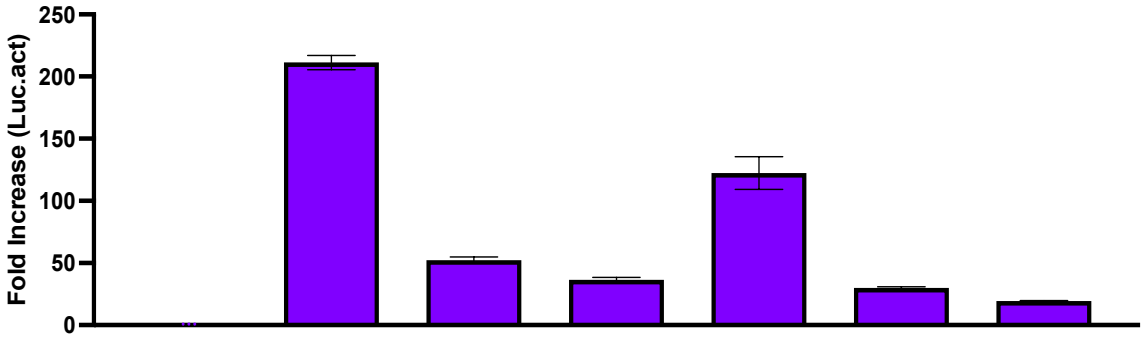
Figure 4. TBHQ interrupts the binding of Nrf2-Keap1. Cells were transfected with HA-Keap1 followed by HA immunoprecipitation and analyzed through western blot. Compared to the first well there is a decrease in Nrf2 in wells treated with 125 µM TBHQ.

Luciferase assay showed no significant effect of Nrf2 activity in the presence of 50 µM and 150 µM TBHQ (Figure 5). Luciferase assay of HEK cells treated with 125 µM TBHQ following the transfection of Nrf2-Myc plasmid showed no further increase in the luciferase activity but instead showed a decrease in the luciferase activity (Figure 6).



Nrf2 (1 µg)	-	+	-	-
TBHQ (µM)	-	-	50	150

Figure 5. TBHQ does not significantly increase the activity of Nrf2. The bars represent means ± SEM, N= 3



Nrf2 (µg)	-	1	0.5	0.1	1	0.5	0.1
TBHQ (125 µM)	-	-	-	-	+	+	+

Figure 6. No effect of TBHQ on luciferase activity after Nrf2 overexpression. The bars represent mean ± SEM, N = 3

Discussion

Initially, we wanted to transfect cells involved in the inflammatory pathway, so we chose BV2 microglia and RAW 264.7 macrophages. BV2 cells are the resident macrophages of the brain and play an important role in the pathology of AD (Hanson et al., 2018). RAW 264.7 cells are derived from BALB/c mice which originate from Abelson leukemia virus transformed macrophages (Taciak et al., 2018). We tried transfecting Nrf2-GFP using various ratios of DNA to transfection reagents, all with unacceptable results. The transfections might have been unsuccessful because the cells are metabolically active and phagocytic which could lead to the plasmid being destroyed when they entered the cell. One method recommended to increase transfection efficiency is through magnetofection which was also attempted but came with unsuccessful results. Thus, these experiments were not presented in this thesis.

After multiple failed attempts of transfection on BV2 and RAW cells, we decided to move on to HEK-293 cells as they have a high transfection efficiency. We initially attempted to overexpress Nrf2-GFP in HEK-293 cells to see if it localizes in the cytoplasm when they are untreated and to determine if TBHQ can activate the Nrf2 pathway leading to the migration of Nrf2 to the nucleus. TBHQ is a food additive and researchers were able to show that TBHQ can reduce oxidative stress in mammals (Zhao et al., 2020). As previously mentioned in the presence of ROS Nrf2 moves into the nucleus and induces the expression of antioxidants which can mitigate oxidative stress. TBHQ has been shown to activate the Nrf2 which can be in response to oxidative stress (Zhao et al., 2020). However, when we overexpressed Nrf2 in HEK-293 cells, Nrf2 localized in the nucleus for both untreated cells and cells treated with TBHQ (Figure 1).

This is likely due to Nrf2 being expressed at such high levels that there is not enough *de novo* synthesis of Keap 1 to bind to the additional Nrf2 and sequester it in the cytoplasm in the absence of TBHQ.

We next attempted to overexpress both Keap 1 and Nrf2 by co-transfecting 293HEK cells with Keap1-HA and Nrf2-GFP. We had hypothesized that overexpression of Keap 1 would provide more Keap 1 to bind to the additional Nrf2 and thus prevent Nrf2 from localizing in the nucleus in untreated cells. While further hypothesized that activation of the Nrf2 pathway by the addition of TBHQ would allow Nrf2 to then localize in the nucleus. Our results did support our hypothesis. Figure 2 reveals that there is more green fluorescence in nucleus of cells treated with TBHQ and more green fluorescence in the cytoplasm of untreated cells. These results further confirm that TBHQ is somehow activating the Nrf2 pathway.

To further confirm that Keap1-HA is interacting with Nrf2-GFP in untreated cells, immunofluorescence was performed on the HA-tag of Keap1. We had hypothesized that the untreated cells would have co-localization of Nrf2 and Keap1 in the cytoplasm, and would be visualized by both red (Keap1) and green (Nrf2) fluorescence overlapping in the cytoplasm. We further hypothesized that treating these cells with TBHQ would eliminate the overlapping fluorescence and reveal that Nrf2 (green) would migrate to the nucleus and Keap1(red) would remain in the cytoplasm. Our results did support these hypotheses (Figure 3). We also noticed that in untreated cells, we often found aggregates of Nrf2 and Keap1 (red and green overlapping fluorescence) in the nucleus. It is possible this occurs simply because there excess Nrf2 and Keap1 that they aggregate through the cell. Also, the nucleus is known to have proteasomes linked to the nuclear pores and thus

able to degrade nuclear proteins. Excess Nrf2 and Keap1 may result in ubiquitination and targeting of Nrf2 to these proteasomes as well. Further studies to determine if the Nrf2-GFP is being ubiquitinated and if so, where in the cell, are possible

To determine if the binding interaction between Keap 1 and Nrf2 is disrupted when cells are treated with TBHQ, HA-tag immunoprecipitation was performed on cells transfected with the Keap1-HA plasmid. We had hypothesized that TBHQ would interrupt the binding between Keap1-HA and native Nrf2, leading to decreasing amounts of Nrf2 associated with the immunoprecipitated Keap1, and would be revealed by in a Western blot. Our preliminary data may support our hypothesis as treatment with TBHQ results in weaker Nrf2 bands. This is most apparent when cells were treated with 125 µM of TBHQ. When compared to untreated cells, 125 µM of TBHQ leads to substantial reduction in Nrf2 at 6 hours, which seems to return 18hrs after treatment. This would mean that in the presence of TBHQ, the Nrf2 pathway is most likely getting activated leading to Nrf2 translocation into the nucleus. Unfortunately, the blot was not reprobed for Keap1 and thus there is no indication that Keap1 was immunoprecipitated. This is a necessary step in all follow up experiments. Additionally, the Nrf2 bands in this preliminary blot are very weak, likely due to there not being enough native Nrf2 to interact with all of the overexpressed Keap1. It is possible that co-transfection with the Nrf2-GFP plasmid would provide more Nrf2 to bind to Keap 1, and the immunofluorescence studies suggest that TBHQ may disrupt this interaction, and thus would be supported in immunoprecipitation studies. Additionally, we could attempt immunoprecipitation of native Keap1. To do this, we would need a polyclonal antibody

for Keap1 in order to maximize antibody binding to the target. We could then use Protein A Sepharose to precipitate the Keap1 and Western blot for Nrf2.

It is important to note that overexpressing Keap1 and Nrf2 leads to a number of problems in interpretation of the data. First, there is no way to control for transfection efficiency unless stable transfectants are isolated. We also noticed that not all cells transfected with one plasmid were also transfected with the second plasmid. This leads the investigator to pick cells that were co-transfected while ignoring others. It is also impossible to control for the amount of Nrf2 or Keap1 that is produced in the transfectant, nor is it possible to choose cells that were all transfected and began expressing the plasmid-encoded protein at the same time. This adds a confounding variable to experiments where treatment time is a variable. Finally, while these experiments are beneficial visually when attempting to understand the Nrf2 pathway, they are not quantitative and not very sensitive, and thus should only be used to supplement more quantitative data.

Initial luciferase assays of BV2 cells transfected with ARE-Luc were unsuccessful, likely due to poor transfection efficiency as stated above for these cells. Thus HEK-293 cells were again chosen for ARE-Luc transfection. Initially we overexpressed ARE-Luc and measured the luciferase expression after TBHQ treatment. After many attempts we did not see any significant increase in the luciferase expression for cells treated with TBHQ. We hypothesized that HEK-293 cells did not express enough Nrf2 or had a low responsiveness to TBHQ. We next decided to overexpress Nrf2 using a plasmid with a coding sequence for Nrf2 with a Myc tag alongside ARE-Luc. We then compared luciferase expression between cells that were only transfected with ARE-

Luc or co-transfected with ARE-Luc and Nrf2-myc, followed by TBHQ treatment. Since TBHQ is a known activator of Nrf2, we hypothesized that cells treated with TBHQ would have a significant increase in the luciferase expression provided there was enough available Nrf2. However, the results did not support our hypothesis. As seen in Figure 5, overexpression of Nrf2 in HEK-293 cells resulted in an increase in luciferase activity, but treatment with TBHQ did not lead to a further increase in luciferase expression. Since we saw an increase in luciferase expression when the cells overexpressed Nrf2, we decided to repeat the experiment by transfecting with different amounts of Nrf2-Myc plasmid followed by 125 µM TBHQ (Figure 6). We hypothesized that cells forced to overexpress less Nrf2 might be more sensitive to TBHQ-induced luciferase production. The results did not support our hypothesis, and in fact there is a decrease in the luciferase expression in the presence of TBHQ (Figure 6). This result is puzzling. TBHQ might be exerting cytotoxic effects on 293HEK cells at the concentrations used. These results suggest that HEK cells might not be the best cell line to study Nrf2 activation because we are unable to stimulate it with any of the known activators.

The current results using the three different plasmids, highlights the need for more research. The original goal of the experiment was to find methods to monitor the Nrf2 pathway. Some of the methods were successful in monitoring the Nrf2 pathway, but are not very sensitive, while others require more research. If any of these methods are deemed successful in monitoring the Nrf2 pathway, they can be used to test potential therapeutic compounds for diseases of oxidative stress. Current research in our lab and others has shown that compounds created by Dr. Green have been shown to act as antioxidants and activate the Nrf2 pathway. Thus, using the plasmids reported here may

provide additional support for the activation of the Nrf2 pathway in a visually appealing way. It would also be beneficial to transfect these plasmids in different cell lines like HT-22 neurons or Caco-2 gastrointestinal epithelial cells as they represent cell types commonly affected by diseases involving oxidative stress, like AD and Celiac disease respectively. Overall, these methods are a good starting point in monitoring the Nrf2 pathway.

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ABSTRACT

ANTIOXIDANT PATHWAY AS POTENTIAL THERAPY FOR ALZHEIMER'S DISEASE

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Oxidative stress is the imbalance between reactive oxygen species and antioxidants in a cell. Often this imbalance is caused by an increase of reactive oxygen species (ROS) leading to dyshomeostasis of the cellular redox balance. Oxidative stress is a major component of several chronic diseases. To mitigate the damage caused by oxidative stress our cells are capable of producing their own antioxidants. One cellular mechanism involves the nuclear factor-erythroid 2-related factor (Nrf2) antioxidant pathway which can be activated in the presence of ROS. To better understand how this pathway works, it is important to track Nrf2 during activation of this pathway. Here we test three different plasmids designed to either force expression of "tagged" proteins in the Nrf2 pathway, or to provide a readout mechanism for the level of Nrf2 activation. These experiments lend support for the efficacy of using these tools to better understand the Nrf2 pathway.